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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

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Process for the preparation of cephradine

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PROCESS FOR THE PREPARATION OF CEPHRADINE

The present invention relates to a process for the preparation of cephradine, and cephradine obtainable by the process according to the invention.

Cephradine is a ß-lactam antibiotic, which may be synthesised in a chemical process, wherein the aminogroup in 7-aminodesacetoxy cephalosporanic acid (7-ADCA) is acylated with a dihydrophenyl glycine side chain. A process for the synthesis of cephradine is for example known from US 3,485,819. For various reasons, the commercially available form of cephradine is a hydrated form in which the water content is in the range of about 3% to 6% of water. This is not a stoichiometric hydrate since the water varies freely in the crystal. An inherent problem of cephradine is its poor stability. By poor stability is meant that cephradine is susceptible to oxidation to cephalexin, to degradation and to coloration. WO 91/00865 discloses a process for the preparation of a more stable cephradine, which process comprises crystallising cephradine hydrate from an aqueous dimethylformamide solution of cephradine. The cephradine thus prepared is a cephradine hydrate stable form (SF), of which the coloration remains essentially the same for a period of at least 8 weeks. By this is meant that the absorbance at 450 nm after 8 weeks is still less than 0.3. The disadvantage of the process WO 91/00865 is that additional foreign substances (dimethylformamide) are necessary to prepare the cephradine hydrate SF with an absorbance at 450 nm of less than 0.3.

The aim of the present invention is to provide a process for the preparation of cephradine hydrate wherein no foreign substances are necessary to obtain cephradine hydrate in a stable form.

The problem is solved according to the invention in that the process comprises:

- reacting 7-amino acid desacetoxy cephalosporanic acid (7-ADCA) with D-dihydrophenylglycine in activated form in the presence of an enzyme in a reaction mixture to prepare cephradine; and
- crystallising the cephradine from an aqueous solution to form cephradine hydrate.

It was surprisingly found that the cephradine hydrate stable form prepared by the process according to the invention has a decreased absorbance measured at a wavelength of 450 nm.

The invention is not limited to a specific cephradine hydrate. Typically, the cephradine hydrate is cephradine monohydrate. The water content of the cephradine hydrate may for instance range between 3% and 6% per weight.

The process according to the invention comprises reacting 7-ADCA with D-dihydrophenylglycine in activated form in the presence of an enzyme in a reaction mixture to prepare cephradine. In the framework of the present invention the wording enzymatic reaction refers to said reacting.

In the process according to the invention the dihydrophenylglycine in activated form may be an amide, for instance a primary, secondary or tertiary amide, or an ester of dihydrophenylglycine. Preferably, the dihydrophenylglycine in activated form is an ester of dihydrophenylglycine, for instance a lower alkyl (1-4 C) ester of dihydrophenylglycine. Preferred is dihydrophenylglycine methyl ester, and most preferred dihydrophenylglycine methylester in the form of a salt of dihydrophenylglycine methyl ester, for example a formic acid or HCl salt of dihydrophenylglycine methylester. The formic acid or HCl salt of the other dihydrophenylglycine esters may also be used.

The molar ratio of dihydrophenylglycine in activated form to 7-ADCA, i.e. the total quantity of dihydrophenylglycine in activated form added (to the reaction mixture) divided to the total quantity of 7-ADCA added (to the reaction mixture), both expressed in moles, may vary between wide limits. Preferably, the molar ratio is below 2.5, preferably between 0.5 and 2.0, and more preferably between 0.7 and 1.8

The reaction mixture may be any suitable mixture in which the reaction of 7-ADCA with D-dihydrophenylglycine in activated form may be carried out in the presence of an enzyme. Preferably, the reaction mixture is an aqueous reaction mixture. The aqueous reaction mixture may also contain an organic solvent or a mixture of organic solvents, preferably less than 30 vol%, more preferably less than 20 vol.%, more preferably less than 10 vol.%, more preferably less than 5 vol.% (relative to the total volume of the liquid). Preferably, the organic solvent is an alcohol with 1-7 carbon atoms, for instance a monoalcohol, in particular methanol or ethanol; a diol, in particular ethylene glycol, or a triol, in particular glycerol. Preferably, the aqueous reaction mixture contains at least 70 vol.% water, more preferably at least 80 vol.%, more preferably at

least 90 vol.%, most preferably at least 95 vol.% water (relative to the sum volume of the liquid).

Depending on the particular reaction conditions, for instance the pH and or the temperature, the cephradine prepared may be present in the aqueous reaction mixture in dissolved form or in the form of cephradine hydrate.

In the process according the invention, the enzymatic reaction may be carried out at any suitable temperature. Preferably, the enzymatic reaction is carried out at a temperature of between -5 and 35°C, preferably between 2 and 30°C. More preferably, the enzymatic reaction is carried out at a temperature of between 5°C and 25°C, and most preferably between 15 and 23°C.

The enzymatic reaction in the process according to the invention, may be carried out at any suitable pH. Preferably, the enzymatic reaction is carried out at a pH of between 6 and 9. More preferably, the enzymatic reaction is carried out at a pH of between 6.3 and 8.5, and most preferably between a pH of 6.5 and 7.5. The pH of the reaction mixture in which the enzymatic reaction is carried out, may be maintained at the desired pH value with any suitable base, for example sodium hydroxide or ammonia.

Any enzyme may be used that is suitable as a catalyst in reacting 7-ADCA with D-dihydromethylglycine in activated form to prepare cephradine. Such enzymes are for instance the enzymes that are known under the general term penicillin acylase, or penicillin G acylase, also called penicillin G amidase or benzylpenicillin acylase (EC 3.5.1.11). Penicillin G acylase refers to a group of hydrolases from microorganisms, especially bacteria, capable of hydrolyzing the 6-acyl group of penicillins or the 7-acyl group of cephalosporins. Microorganisms from which penicillin acylase enzymes may be obtained are for example *Acetobacter*, in particular *Acetobacter pasteurianum*, *Aeromonas*, *Alcaligenes*, in particular *Alcaligenes faecalis*, *Aphanocladium*, *Bacillus sp.*, in particular *Bacillus megaterium*, *Cephalosporium*, *Escherichia*, in particular *Escherichia coli*, *Flavobacterium*, *Fusarium*, in particular *Fusarium oxysporum* and *Fusarium solani*, *Kluyvera*, *Mycoplana*, *Protaminobacter*, *Proteus*, in particular *Proteus rettgari*, *Pseudomonas* and *Xanthomonas*, in particular *Xanthomonas citrii*.

In a preferred embodiment, the enzyme used in the process according to the invention is a mutant penicillin acylase. Mutants of penicillin acylases can be made by starting from any known penicillin acylase. The mutated acylases which are used in the process according to the invention, are for example derived from wild type acylases via recombinant DNA methodology known in the art, by substituting one amino acid residue

for a new residue. Mutant penicillin acylases are for example penicillin acylases, wherein one or more amino acid residues have been replaced compared to the wild-type enzyme, and the mutant penicillin acylase has altered substrate specificity and/or altered enzymatic activity. An example of mutant penicillin acylase with altered substrate specificity is mutant Phe24-Ala which is known from WO 98/20210. The numbering of the position of the amino acids corresponds to the numbering of the amino acid sequence of wild type Penicillin G acylase of *E. coli*.

In another preferred embodiment of the invention the enzyme is immobilised on a carrier. In immobilised form the enzyme can be readily separated and recycled. Immobilised enzymes are known as such and are commercially available, for example an Escherichia coli penicillin acylase isolated as decribed in WO 92/12782 and immobilised as described in EP 222 462 and in WO97/04086

The preferred conditions for the enzymatic reaction for the preparation of cephradine are also applicable without necessarily applying the crystallisation according to the invention.

The process according to the invention comprises crystallising the cephradine from an aqueous solution to form cephradine hydrate. The aqueous solution comprising the cephradine that is prepared in the enzymatic reaction may be obtained in any suitable way. The aqueous solution comprising the cephradine may for example be the aqueous reaction mixture. The aqueous solution comprising the cephradine may also be prepared by dissolving cephradine hydrate that may have been formed in the presence of said enzyme. Preferably, the enzyme is separated from the aqueous solution prior to crystallising cephradine hydrate.

Cephradine hydrate may be dissolved in any suitable way. Dissolving cephradine hydrate may for example be performed at a pH at or above 8, more preferably, at a pH of between 8.3 and 9.5, and most preferably at a pH of between 8.5 and 9. In a preferred embodiment, dissolving cephradine hydrate is for example performed by modifying, in particular by increasing the pH of the reaction mixture to a value at or above 8, preferably, at a pH of between 8.3 and 9.5, and most preferably at a pH of between 8.5 and 9. The pH of the reaction mixture may be increased at the desired pH value by addition of any suitable base, for example sodium hydroxide or ammonia. Said dissolving may be carried out batch-wise or continuously. It is also possible to separate cephradine hydrate from the reaction mixture, and to dissolve the separated cephradine hydrate to form the aqueous solution.

The aqueous solution may be any suitable solution comprising dissolved cephradine. The aqueous solution may also contain an organic solvent or a mixture of organic solvents, preferably less than 30 vol%, more preferably less than 20 vol.%, more preferably less than 10 vol.%, more preferably less than 5 vol.% (relative to the total volume of the liquid). Preferably, the organic solvent is an alcohol with 1-7 carbon atoms, for instance a monoalcohol, in particular methanol or ethanol; a diol, in particular ethylene glycol, or a triol, in particular glycerol. Preferably, the aqueous solution contains at least 70 vol.% water, more preferably at least 80 vol.%, more preferably at least 90 vol.%, most preferably at least 95 vol.% water (relative to the sum volume of the liquid).

Preferably, the enzyme is separated from the cephradine prepared. The enzyme may for example be separated from the reaction mixture comprising the cephradine, for instance by sieving the reaction mixture over a sieve to separate the enzyme from the cephradine.

Separating the enzyme may also be carried out after dissolving cephradine hydrate that may have been prepared during the enzymatic reaction. Separating the enzyme from an aqueous solution comprising dissolved cephradine, may for example be performed according to any method known in the art, such as filtration or centrifugation. Preferably, separating the enzyme is performed prior to crystallising cephradine.

Crystallisation of cephradine prepared in the process according to the invention may be carried out in any suitable way. Preferably, said crystallising is performed at a temperature between 45 and 60°C, preferably between 48 and 55°C. Most preferably, crystallising is performed at a temperature between 49 and 52°C.

Crystallising cephradine from an aqueous solution comprising cephradine may be performed at any suitable pH. Preferably, crystallising the cephradine may be performed at a pH of between 4.0 and 6.0, preferably at a pH of between 4.5 and 5.5, more preferably at a pH of between 4.7 and 5. In the framework of the invention, the pH of the aqueous solution may be lowered in several ways, for instance chemically, by adding an acid, for instance a mineral acid, in particular sulphuric acid, hydrochloric acid or nitric acid. Preferably, said crystallising is performed continuously.

Working within the preferred temperature and pH ranges, it was surprisingly found to that the process results in cephradine hydrate with strongly decreased absorbance at 450 nm. Therefore, the invention also relates to a process for preparing cephradine hydrate crystals, characterised in that the process comprises crystallising cephradine hydrate from a solution containing dissolved cephradine at a temperature

between 45 and 60°C, preferably between 48 and 55°C. The pH is preferably between 4.0 and 6.0, more preferably between 4.5 and 5.5, most preferably between 4.7 and 5.

The process according to the invention also comprises performing said crystallising at such pH and at such temperature that the absorbance at 450 nm of the cephradine hydrate prepared is below 0.050, preferably below 0.040, and most preferably below 0.030, usually above 0.005.

In one embodiment of the process according to the invention the enzymatic reaction is carried out in the presence of sodium bisulphite. Preferably, the amount of sodium bisulphite present in the enzymatic reaction is between 1 and 25 mM, preferably between 5 and 15 mM. Surprisingly, the presence of sodium bisulphite during the enzymatic reaction further decreased the coloration of the cephradine prepared.

Sodium bisulphite may also be present during crystallisation of cephradine in the process according to the invention. Preferably, the amount of sodium bisulphite present during crystallising of cephradine to form cephradine monhydrate crystals is between 5 and 250 mM, more preferably between 25 and 150 mM.

The cephradine hydrate may be separated from the aqueous solution and dried in any suitable manner.

In another aspect, the invention relates to cephradine hydrate obtainable by the process as described herein. Surprisingly, the coloration of the cephradine hydrate obtainable by the process according to the invention is low, which means that the absorbance at 450 nm is below 0.050, preferably the absorbance at 450 nm is between 0.005 and 0.050, and more preferably of between 0.008 and 0.040, and most preferably between 0.010 and 0.030. It was also found that the cephradine hydrate obtainable by the process according to invention, shows a low coloration in the stress stability test, i.e. the coloration of the cephradine hydrate is less than 0.20, more preferably below 0.15, and most preferably below 0.10 after 8 weeks.

In another embodiment, the invention comprises cephradine hydrate with an absorbance at 450 nm of below 0.05, preferably of between 0.005 and 0.05, and more preferably of between 0.010 and 0.040.

Preferably, the cephradine hydrate prepared according to the process of the invention contains no, or substantially no dimethylformamide.

The following example is illustrative for the invention, without limiting the invention thereto.

EXAMPLES

Example 1

a) Enzyme and Immobilisation

The penicillin acylase as used herein was a Pen-G acylase mutant Phe-24-Ala, as described in WO 98/20120. The enzyme was immobilised as described in EP 222 462, with gelatin and chitosan being used as gelling agent and glutaraldehyde as cross-linker.

b) Synthesis of cephradine

An enzyme reactor (100 ml), with a sieve bottom with 175 µm gauze was filled with 40 g nett-wet immobilised Pen-G acylase mutant F24A (enzyme loading 40 mg/ g nett-wet biocatalyst). Then, 110.0 ml water (20 °C), 0.3 g sodium bisulphite, 36.6 g 7-ADCA (169.8 mmol), 1 ml 25 % NH_4OH solution and 0.04 g EDTA were added. The suspension was stirred for 5 minutes at T = 20 °C. The pH was 6.90.

In a separate vessel 37.8 g dihydrophenylglycine methylester.HCl salt (DHME; 174.7 mmol) was dissolved in 67.2 ml water at T = 20 °C. From t = 0 to t = 60 min this solution was dosed into the enzyme reactor with constant dosing rate. The temperature was kept at T = 20 °C. The pH was kept at 6.90 with 25 % NH_4OH solution. In the second part of the reaction the pH slowly increased: t = 0-240 min: pH = 6.90; t = 270 min: pH = 7.00; t = 350 min: pH = 7.10.

c) Crystallisation and isolation of cephradine.

The reaction mixture obtained as described above was used for crystallisation and isolation. At t= 350 minutes (from the start of cephradine synthesis, see above), the mixture comprising the cephradine was cooled to 3 °C and a suspension of 1.8 g sodium bisulphite in 4.7 ml water was added. Then, the pH was increased to 8.6 with 25 % NH_4OH solution.

At t = 360 minutes, the enzymatic reactor was discharged via the sieve bottom. The (immobilised) enzyme wetcake on the sieve was washed with 2 x 30 ml water (2 °C). The filtrates and washings were combined and filtrated (successively through filters with pore size 40 µm, 10 µm and 3 µm).

A crystallisation reactor was charged with 3.0 g cephradine and 50 ml water and heated up to T = 52 °C. Immediately, the combined filtrates and washings were dosed with constant dosing rate in 60 minutes . The temperature was kept at T= 52 °C and the pH at 4.80 by titration with 25 % sulfuric acid. Then, the temperature was decreased to 25 °C in 30 minutes. The resulting suspension was filtered through a glass filter. The wetcake was washed with 30 ml water and 2 x 25 ml 80 % acetone (acetone/water = 80/20 v/v) and dried. 47.0 g cephradine hydrate, with a water content of 3.4 %, was obtained.

d) Coloration of cephradine

The coloration of cephradine hydrate was determined by the absorbance at 450 nm. The absorbance and stress stability of the cephradine hydrate prepared as described above were determined.

Absorbance

1 g of cephradine hydrate was dissolved in 10 ml 10 % aqueous sodium carbonate solution. The absorbance was determined at 450 nm (= A_{450}) on a Perkin Elmer 550 S spectrophotometer, with 10 % aqueous sodium carbonate solution as a reference solution at room temperature. The A_{450} of cephradine hydrate prepared as described above was 0.012.

Stress stability test

Cephradine hydrate was kept at 40°C at a relative humidity of 75%. After 0, 2, 4 and 8 weeks the absorbance at 450 nm was determined as described above. Table 1 shows that after 8 weeks the absorbance of the cephradine hydrate obtained as described above was still below 0.10.

Table 1. Stress stability cephradine hydrate

Week	A_{450}
0	0.025
2	0.043
4	0.058
8	0.096

CLAIMS

1. Process for the preparation of cephradine characterised in that the process comprises:
   - reacting 7-aminodesacetoxy cephalosporanic acid (7-ADCA) with D-dihydrophenylglycine in activated form in the presence of an enzyme in a reaction mixture to prepare cephradine;
   - crystallising the cephradine from an aqueous solution to form cephradine hydrate.

2. Process according to claim 1, characterised in that the process comprises preparing said aqueous solution by dissolving cephradine hydrate.

3. Process according to claims 1 to 2, characterised in that dihydrophenylglycine in activated form is dihydrophenylglycine methylester.

4. Process according to any one of the claims 1 to 3, characterised in that the dihydrophenylglycine in activated form is a HCL salt of dihydrophenylglycine methylester.

5. Process according to any one of the claims 1 to 4, characterised in that said reacting is carried out at a temperature between 2 and 30°C.

6. Process according to any one of the claims 1 to 5, characterised in that said reacting is carried out at a temperature, between 5 and 25°C.

7. Process according to any one of the claims 1 to 6, characterised in that said reacting is carried out at a pH of between 6 and 9.

8. Process according to any of the claims 1 to 7, characterised in that said reacting is carried out at a pH between 6.3 and 8.5.

9. Process according to any one of the claims 1 to 8, characterised in that the enzyme is a penicillin acylase.

10. Process according to any one of the claims 1 to 9, characterised in that the enzyme is a mutant penicillin acylase.

11. Process according to any one of the claims 1 to 10, characterised in that the enzyme is immobilised on a carrier.

12. Process according to any one of the claims 1 or 11, characterised in that the process comprises separating the enzyme from the cephradine prior to said crystallising.

13. Process according to any one of the claims 1 or 12, characterised in that said dissolving is performed at a pH above 8.

14. Process according to any one of the claims 1 to 13, characterised in that said dissolving is performed at a pH between 8.5 and 9.

15. Process for preparing cephradine hydrate crystals, characterised in that the process comprises crystallising cephradine from an aqueous solution to form cephradine hydrate, wherein said crystallizing is carried out at a temperature of between 45 and 60°C, preferably between 48 and 55°C.

16. Process according to any one of the claims 1 to 14, characterised in that said crystallising is performed at a temperature of between 45 and 60°C, preferably between 48 and 55°C.

17. Process according to any one of the claims 1 to 16, characterised in that said crystallising is performed at a pH between 4.0 and 6.0, preferably at a pH between 4.5 and 5.5.

18. Process according to any one of the claims 1 to 17, characterised in that said crystallising is performed at such pH and at such temperature that the absorbance at 450 nm of the cephradine hydrate prepared is below 0.050.

19. Process according to any one of the claims 1 to 18 characterised in that reacting 7-aminodesacetoxy cephalosporanic acid (7-ADCA) with D-dihydrophenylglycine in activated form in the presence of an enzyme in a reaction mixture to prepare cephradine is carried out in the presence of sodium bisulphite.

20. Cephradine hydrate obtainable by the process according to any one of the claims 1 to 19.

21. Cephradine hydrate with an absorbance at 450 nm of less than 0.05.

ABSTRACT

The present invention describes process for the preparation of cephradine hydrate characterised in that the process comprises:

- reacting 7-amino acid desacetoxy cephalosporanic acid (7-ADCA) with D-dihydrophenylglycine in activated form in the presence of an enzyme in a reaction mixture to prepare cephradine;
- crystallising the cephradine from an aqueous solution to form cephradine hydrate.

The invention further describes cephradine hydrate obtainable by a process according to invention. The invention also describes cephradine hydrate with an absorbance at 450 nm of below 0.05.